AD_____

Award Number: DAMD17-00-1-0618

TITLE: Isolation and Analysis of Tubulin Carboxypeptidase: A Chemotherapeutic Target Arising from Tubulin Tyrosine Ligase Suppression in Human Breast Tumors

PRINCIPAL INVESTIGATOR: Robert L. Margolis, Ph.D.

CONTRACTING ORGANIZATION: CNRS
France

REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (15 Sep 00 - 14 Sep 01)

4. TITLE AND SUBTITLE
Isolation and Analysis of Tubulin Carboxypeptidase: A Chemotherapeutic Target Arising from Tubulin Tyrosine Ligase Suppression in Human Breast Tumors

5. FUNDING NUMBERS
DAMD17-00-1-0618

6. AUTHOR(S)
Robert L. Margolis, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CNRS
France

E-Mail: saudraix@ibs.fr

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Microtubules are critical to accurate completion of mitosis. In mammalian cells, the microtubule subunit, tubulin, undergoes a cyclic modification by removal and subsequent addition of tyrosine on the α-subunit C-terminus. This process uses two specific enzymes, tubulin tyrosine ligase (TTL) and tubulin carboxypeptidase (TCP). We have recently demonstrated that suppression of this cycle may play an important role in tumor development. TTL is frequently and specifically suppressed during tumor growth in situ, suggesting a tumor suppressor function. In the complete absence of TTL activity TCP can cleave the second C-terminal residue of α-tubulin, yielding a truncated tubulin designated "Δ2-tubulin". Due to TTL suppression, Δ2-tubulin uniquely appears in tumor cells. This specific marker has implications for early breast tumor detection. Further, if suppression of TTL strongly enhances tumor growth, these findings may permit chemotherapeutic intervention through suppression of the TCP to restore the normal tubulin status. We intend to isolate and sequence human TCP as a first step toward determining if suppression of TCP may serve as a unique chemotherapy target.

14. SUBJECT TERMS

15. NUMBER OF PAGES
16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

DAMD17-00-1-0618

Robert L. MARGOLIS

Isolation and analysis of tubulin carboxypeptidase: a chemotherapeutic target arising from tubulin tyrosine ligase suppression in human breast tumors.

Introduction

Microtubules are critical to accurate completion of mitosis. Further, our recent work has also implicated microtubules in cell cycle progression through the G1 phase of the cell cycle. In all mammalian cells, the constituent subunit of microtubules, tubulin, undergoes a cyclic modification by removal and subsequent addition of tyrosine on the C-terminus of the alpha subunit, using two specific enzymes, a tubulin tyrosine ligase (TTL) and a tubulin carboxypeptidase (TCP). Although highly conserved in evolution, the function of this unique modification has remained unknown. We have recently demonstrated that suppression of this cycle may play an important role in tumor development. From several lines of evidence, we are able to conclude that the enzyme that adds tyrosine to the C-terminus of tubulin, TTL, is frequently and specifically suppressed during tumor growth in situ, suggesting a possible tumor suppressor function. For example, a survey of human breast tumor biopsies has shown that both a pool of 100 tumors and the majority (6/7) of individual breast tumor biopsies show suppression of TTL activity, whereas such suppression never occurs in normal tissue. As a result of suppression of TTL activity, a unique antigen that is never present in normal tissues, Δ2-tubulin appears in tumor cells. The presence of this specific marker has implications for early breast tumor detection. Further, if suppression of TTL strongly enhances tumor growth, as we suspect, our approach may permit unique chemotherapeutic intervention through specific suppression of the tubulin carboxypeptidase to restore the normal tubulin status.

BODY:

There were no tasks indicated in the original application for this Concept Award, in which the concept was defined in one paragraph.

We have set out to establish parameters for the assay and purification of tubulin carboxypeptidase. As there has been no background work in this area to date, we had to establish some very basic information that would enable the purification and assay of this protein.

We have, in the past year, established that the tubulin carboxypeptidase is active in interphase cells, but is apparently silenced during mitosis. We used immunofluorescence confocal microscopy to determine that microtubules of the HeLa mitotic spindle remain tyrosinated during prolonged mitotic arrest in the presence of taxol, which arrests cells in mitosis with a fully formed but inactive mitotic spindle. In contrast, the continuing activity of tubulin carboxypeptidase during drug treatment caused microtubules in surrounding interphase cells to become fully detyrosinated, as determined by antibodies specific to the tyrosinated and detyrosinated forms of tubulin. Similar results were obtained in an NIH3T3 cell that we have shown lacks tubulin tyrosine ligase activity (Lafanechère L, Courtay-Cahen C, Kawakami T, Jacrot M, Wehland J, Job D and Margolis RL, 1998, Suppression of tubulin tyrosine ligase during tumor growth. J Cell Sci 111, 171-181). In these cells, we found that, whereas microtubules are largely detyrosinated as a result of the lack of activity of the tubulin tyrosine ligase enzyme, mitotic spindle microtubules were largely composed of the tyrosinated form of tubulin (FIGURE 1). This result indicated that tubulin carboxypeptidase was inactive in the mitotic cells, and that the tyrosinated form of tubulin (which is the form that exists prior to posttranslational modification) predominated.

These results gave the promise of an assay that could be used to establish that we had indeed isolated the correct carboxypeptidase enzyme, as the correct activity should be inactivated by mitotic extract or directly by cdc2 protein kinase activity, or absent in mitotic extracts from the same cells which yield an activity in interphase extracts. This capacity to distinguish the activity from other contaminating carboxypeptidases is important, as multiple

carboxypeptidases are expressed in cells and there had previously been no clear way of distinguishing the activity for which tubulin is the physiological substrate from contaminating or irrelevant activities. The physiological meaning of mitotic silencing of the tubulin carboxypeptidase remains for future work to establish.

We then turned our attention to establishing an in vitro assay for tubulin carboxypeptidase activity. Such an assay would determine the extent of tubulin detyrosination. The assay we established used fully tyrosinated tubulin, isolated from interphase HeLa cells, and then assembled into microtubules that were stabilized with taxol. For this, we found the conditions to assemble tubulin from HeLa cell extracts, and established that the isolated microtubules reacted with antibodies to tyrosinated tubulin, but not to antibodies directed against detyrosinated tubulin (FIGURE 2, lane 3). The isolation protocol that we used employed assembly of the tubulin into microtubules using taxol to stabilize the assembled state, and then centrifuging the after a preclearing centrifugation step prior to assembly. Only taxol stabilized microtubules pelleted under these conditions.

As the crude extract microtubules were entirely tyrosinated, they were excellent substrates for assay of tubulin carboxypeptidase activity. The activity could be followed by centrifuging taxol stabilized microtubules in the course of the reaction, followed by western blot analysis of the detyrosinated status of the tubulin present in the microtubule pellets (FIGURE 2, lanes 4 and 5). Nocodazole incubation of extract aliquots prevented assembly of microtubules and served as a control. Pellets contained no microtubules, and no signal for either tyrosinated (not shown) or detyrosinated (shown) tubulin.

The first thing we were able to establish with this in vitro assay of microtubule detyrosination was that our predication was correct that mitotic extracts contained no activity capable of detyrosinating microtubules in vitro, while interphase extracts reproducibly had this activity (FIGURE 2, lanes 4 and 5).

Using the in vitro assay of tubulin carboxypeptidase activity, as described above, we have begun to establish isolation procedures for the tubulin

carboxypeptidase. In preliminary work in this direction, we have found that the enzyme bound specifically to assembled microtubules in vitro, and that microtubule capture could be used as a procedure for partial purification of the enzyme.

We have then established conditions for the separation of the enzyme activity from microtubules in vitro. The separation is important for further purification, as it permits column isolation of enzyme activity after separation from microtubules. Our further work has been hampered to some degree by the fact that the carboxypeptidase activity is somewhat labile. We are therefore in the process of defining conditions that will permit more rapid isolation and analysis, and buffer conditions that will stabilize the activity, so that we can overcome this technical hurdle.

In other work completed during this period on the subject, we copublished a report, in collaboration with several other laboratories, that detyrosination of tubulin may be used as a marker of the degree of to define high- and low- risk groups in breast cancer tumor. Our study further showed that tubulin detyrosination is a frequent occurrence in breast cancer, easy to detect and linked to tumor aggressiveness. Tubulin-carboxypeptidase inhibitors could therefore potentially suppress tubulin detyrosination in cancer cells and thereby impede tumor progression (Mialhe A, Lafanechère L, Treilleux I, Peloux N, Dumontet C, Brémond A, Panh MH, Payan R, Wehland J, Margolis RL and Job D., 2001, Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. Cancer Res 61, 5024-5027). This published report cites funding from the DOD (see Appendix).

The published findings are important to interpretation of the utility of further work to purify the tubulin carboxypeptidase enzyme. It is clear that the enzyme has the potential to be an important target for chemotherapy of advanced breast tumors. Beyond the one year DOD funding period, which has terminated, we will therefore continue our work to identify and sequence tubulin carboxypeptidase, ultimately for use in high throughput screens to identify inhibitory compounds.

FIGURE 1

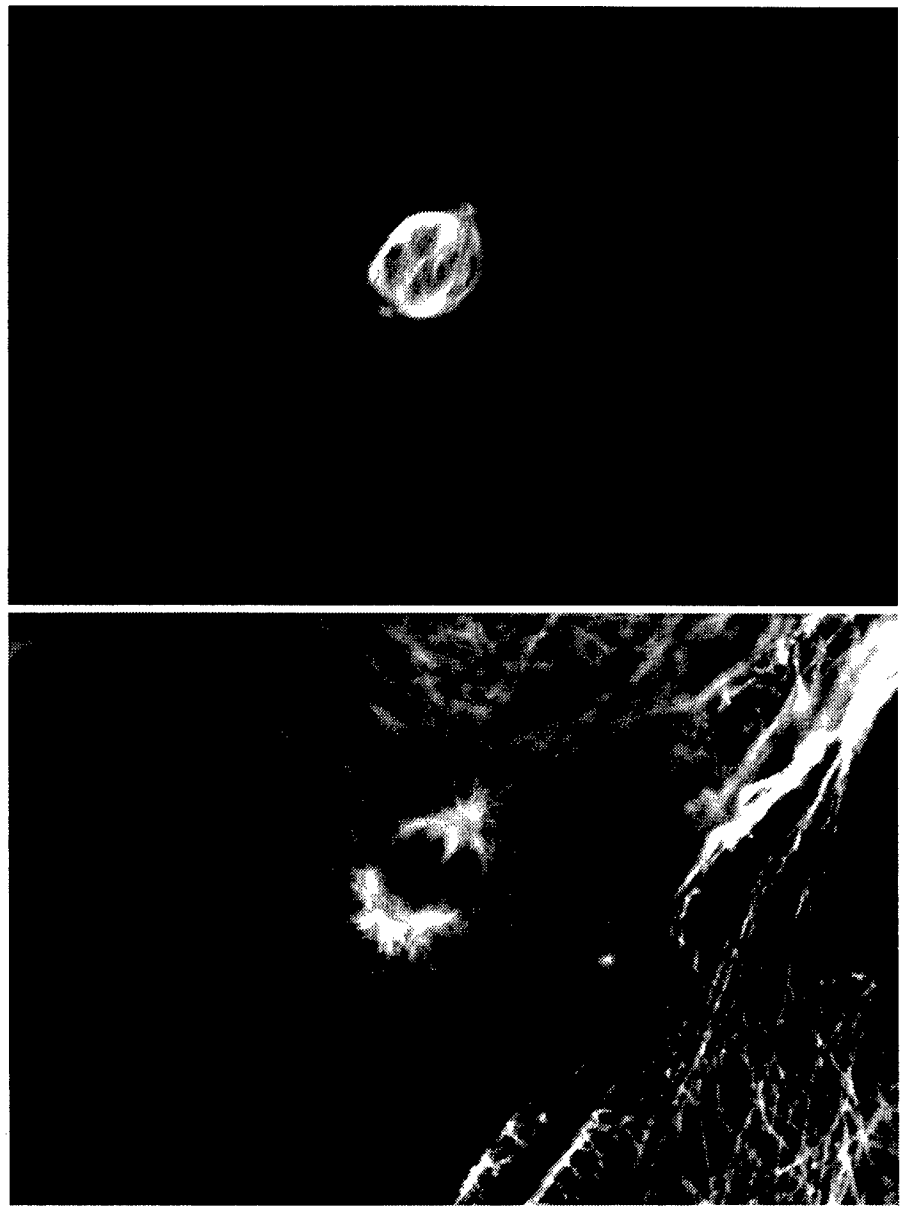

Figure 1. Immunofluorescence analysis of tubulin tyrosination in NIH3T3 cells that were null for tubulin tyrosine ligase. Both mitotic and interphase cells are positive for detyrosinated tubulin microtubules, (B); whereas only mitotic spindles were positive for the presence of tyrosinated tubulin (A) and interphase microtubule arrays were not.

FIGURE 2

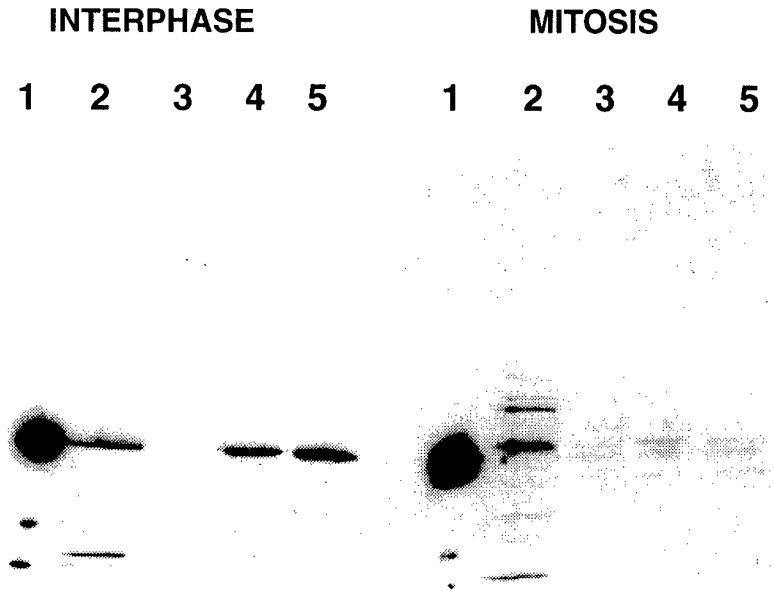

Figure 2. Assay of tubulin carboxypeptidase activity on in vitro isolated microtubules and demonstration that the activity is absent in mitosis. HeLa cell cytosolic extracts were made for both mitotic and interphase cells. Mitotic cells were obtained by block of the cells with nocodazole in mitosis for 24 hr. followed by a shakeoff to obtain a pure mitotic population. Following sonication and centrifugation to obtain a cytosolic supernatant, aliquots were incubated with either nocodazole or taxol, then assayed for the presence of detyrosinated tubulin. Lanes 1, bovine brain tubulin as a control. Lanes 2, initial high speed supernatant; Lanes 3, initial high speed pellet; Lanes 4, high speed pellet after 30 min incubation at 37° C in the presence of taxol; Lanes 5, high speed pellet after 60 min incubation in taxol. It is evident that extract microtubules have little detyrosinated tubulin, either in mitosis or interphase (Lanes 3). Whereas interphase microtubules become progressively detyrosinated during incubation (Lanes 4-5, left), mitotic microtubules have no evidence of activity (Lanes 4-5 right). The major band is tubulin, as made evident by the bovine brain tubulin control (Lanes 1).

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstrated that tubulin carboxypeptidase is active in interphase cells, but is apparently silenced during mitosis.
- Established that tubulin isolated from interphase HeLa cells is fully tyrosinated and therefore useful for in vitro activity assays.
- We have found that tubulin carboxypeptidase bound specifically to assembled microtubules in vitro, and that microtubule capture could therefore be used as a procedure for partial purification of the enzyme.
- Demonstrated that tubulin detyrosination is a frequent occurrence in breast cancer, easy to detect and linked to tumor aggressiveness. This work lends importance to the search for tubulin carboxypeptidase inhibitors (collaborative project, paper in Appendix).

REPORTABLE OUTCOMES:

None to date.

CONCLUSIONS:

We have made a reasonable start in the process of determining the conditions for the isolation, purification and sequencing of tubulin carboxypeptidase. We have conducted similar work in the past on other proteins such as STOP, TD-60 and CENP-A. Our progress to date is similar to the progress we have made in similar periods of time in previous isolation projects. The most difficult work is to establish reliable assay protocols and to establish that one can obtain meaningful amounts of protein for assay. This work is now largely completed, and we are in a good position to conduct the complete isolation of the protein.

"So what" –

We have previously demonstrated that tubulin carboxypeptidase is a reasonable candidate for a tumor suppressor protein in breast tumors. With our recently published results demonstrating that tubulin detyrosination is a frequent occurrence in actual human breast cancers and is linked to tumor

aggressiveness, our efforts to obtain the protein, and then search for suppressors of its activity, should be important for breast cancer treatment protocols.

Further, the big surprise that tubulin carboxypeptidase is silenced in mitosis indicates that the reason it is a tumor suppressor does not relate to mitotic function. This was unexpected for a microtubule modifying protein that could be reasonably expected to influence mitosis. We have previously shown (Trielli MC, Andreassen PR, Lacroix FB and Margolis RL., 1996, Differential microtubule dependent arrest of transformed and non-transformed cells in the G1 phase of the cell cycle, and specific related mortality of transformed cells. J Cell Biol 135, 689-700) that microtubules are also very important to G1 progression, so we are turning our attention to the possibility that tubulin carboxypeptidase is involved in control of G1 progression. This would place it in company of a number of other known tumor suppressors that act on G1 cell cycle controls.

REFERENCES:

Lafanechère L, Courtay-Cahen C, Kawakami T, Jacrot M, Wehland J, Job D and Margolis RL, 1998, Suppression of tubulin tyrosine ligase during tumor growth. J Cell Sci 111, 171-181.

Mialhe A, Lafanechère L, Treilleux I, Peloux N, Dumontet C, Brémond A, Panh MH, Payan R, Wehland J, Margolis RL and Job D., 2001, Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. Cancer Res 61, 5024-5027.

Trielli MC, Andreassen PR, Lacroix FB and Margolis RL., 1996, Differential microtubule dependent arrest of transformed and non-transformed cells in the G1 phase of the cell cycle, and specific related mortality of transformed cells. J Cell Biol 135, 689-700.

APPENDICES:

One published paper:

Mialhe A, Lafanechère L, Treilleux I, Peloux N, Dumontet C, Brémond A, Panh MH, Payan R, Wehland J, Margolis RL and Job D., 2001, Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. Cancer Res 61, 5024-5027

[CANCER RESEARCH 61, 5024–5027, July 1, 2001]

Tubulin Detyrosination Is a Frequent Occurrence in Breast Cancers of Poor Prognosis[1]

Agnes Mialhe,[2] Laurence Lafanechère,[2] Isabelle Treilleux, Nadine Peloux, Charles Dumontet, Alain Brémond, Meng-Hong Panh, Raoul Payan, Jürgen Wehland, Robert-Louis Margolis, and Didier Job[3]

Laboratoire du Cytosquelette, Institut National de la Santé et de la Recherche Médicale (INSERM) U366, Département de Biologie Molèculaire et Structurale, Commissariat á l'Energie Atomique, 38054 Grenoble Cedex 9, France [A. M., L. L., D. J.]; Département d'Anatomie et de Cytologie Pathologiques, Centre Léon Bérard, 69373 Lyon, France [I. T., N. P., C. D., A. B.]; Clinique Belledonne, 38400 Saint Martin d'Hères, France [M. H. P., R. P.]; Gesellschaft für Biotechnologische Forschung mbH, 38092 Braunschweig, Germany [J. W.]; and Institut de Biologie Structurale J. P. Ebel (Commissariat á l'Energie Atomique-Centre National de la Recherche Scientifique), 38027 Grenoble Cedex 1, France [R. L. M.]

ABSTRACT

Tubulin, the dimeric subunit of microtubules, is a major cell protein that is centrally involved in cell division. Tubulin is subject to specific enzymatic posttranslational modifications including cyclic tyrosine removal and addition at the COOH terminus of the α-subunit. Tubulin is normally extensively tyrosinated in cycling cells. However, we have previously shown that detyrosinated tubulin accumulates in cancer cells during tumor progression in nude mice. Tubulin detyrosination, resulting from suppression of tubulin tyrosine ligase and the resulting unbalanced activity of tubulin-carboxypeptidase, apparently represents a strong selective advantage for cancer cells. We have now analyzed the occurrence and significance of tubulin detyrosination in human breast tumors. We studied a total of 134 breast cancer tumors from patients with or without known complications over a follow-up period of 31 ± 10 months. The mean age of the patients at the time of diagnosis was 57 years. For each patient, detailed data concerning the histology and extension of the tumor were available. Tumor cells containing detyrosinated tubulin were visualized by immunohistochemical staining of paraffin-embedded tissue sections.

Cancer cells with detyrosinated tubulin were observed in 53% of the tumors and were predominant in 19.4% of the tumors. Tubulin detyrosination correlated to a high degree of significance ($P < 0.001$) with a high Scarf-Bloom-Richardson (SBR) grade, a known marker of tumor aggressiveness. Among SBR grade 1 tumors, 3.8% were strongly positive for tubulin detyrosination compared with 65.4% of the SBR grade 3 tumors. The SBR component showing the strongest correlation with tubulin detyrosination was the mitotic score. In the entire patient population, neither the SBR grade nor the detyrosination index had significant prognostic value ($P = 0.11$, $P = 0.27$, respectively), whereas a combined index was significantly correlated with the clinical outcome ($P = 0.02$). A preliminary subgroup analysis indicated that tubulin detyrosination may define high- and low- risk groups in breast cancer tumors with an SBR grade of 2. Our study shows that tubulin detyrosination is a frequent occurrence in breast cancer, easy to detect, and linked to tumor aggressiveness.

INTRODUCTION

Microtubules are major cytoskeletal structures, centrally involved in the control and mechanics of cell division, being the principal components of the mitotic spindle in eukaryotic cells (1). The building block of microtubules is the αβ-tubulin heterodimer. Tubulin is subject to specific posttranslational modifications including a cycle of tyrosine removal and addition at the COOH terminus of the α subunit (2, 3). This cycle involves two enzymes, TTL[4] (4) and an ill-defined tubulin carboxypeptidase (5, 6), and generates two major forms of tubulin: tyrosinated tubulin (Tyr-tubulin) and Glu-tubulin. A third tubulin species (Δ2-tubulin) arises by removal of the COOH-terminal Glu residue from the α chain of Glu-tubulin (7, 8). Tyr-tubulin is the dominant tubulin species in cycling cells (9, 10). Glu-tubulin is abundant in neurons but can be present in stable microtubules of other cell types (10–14). Δ2-tubulin normally has high neuronal specificity (7, 8, 13, 15). However, we have previously observed an abnormal accumulation of Glu-tubulin and Δ2-tubulin in cancer cells of both fibroblastic and epithelial origin, during tumor growth in nude mice (15). This accumulation is attributable to TTL suppression and apparently represents a strong selective advantage for cancer cells (15). Here, we have tested whether accumulation of Glu- and Δ2-tubulin also occurred in human tumor cells and whether tubulin detyrosination was related to tumor severity.

MATERIALS AND METHODS

Patient Population. Entered in this study were tumor samples from 134 breast cancer patients treated at the Center Léon Bérard, between January 1996 and December 1998. The end point for clinical follow-up was December 31, 1999. All of the tumors were primary breast tumors. The study included tumors from all of the patients who had locoregional recurrence, metastasis, or death during the period of follow-up. Among these tumors, tumors <40 mm in size ($n = 34$) were paired with tumors of the same size from patients of the same age with no event. Tumors ≥40 mm in size ($n = 18$) could not be paired because of difficulties in finding subjects of the same age in the relatively small-sized population of patients with similar tumors and no event ($n = 48$). All of these 48 patients were included in the study. The median follow-up (from diagnosis of the tumor to December 1999) was 32.2 months (range, 12.2–48.2 months). All of the patients were seen on a regular basis, every 6 months. The principal patient characteristics are described in Table 1.

Tumor samples were collected at the Department of Pathology of the Center Léon Bérard. Tumors were pure histological variants of invasive breast carcinomas comprising 100 ductal, 15 lobular, and 19 other variants (including mucinous, tubular, and metaplastic). The mean largest diameter of collected tumors was 37.4 mm (range, 2–100 mm; Table 1). The histopathological type and grade of the carcinomas were evaluated according to WHO criteria and SBR grading, respectively (16). The SBR grade includes three histological parameters: tubular differentiation, nuclear pleiomorphism, and mitotic count.

Immunohistochemistry of Paraffin-embedded Tissue. The cellular content in Glu-tubulin and in Δ2-tubulin was evaluated by immunohistochemical analysis, using specific antibodies. Tumor samples were Bouin-fixed within 2 h after surgical removal. Sections of 4 μm in thickness from paraffin-embedded blocks were deparaffinized in xylene and rehydrated in a decreasing ethanol series (100 to 50%). At this stage, tissue sections assigned to Δ2-tubulin immunohistochemistry were treated for antigen retrieval. Antigen retrieval involved treatment in a sodium citrate solution [10 mM (pH 6.0)] in a 700-W microwave oven, three times for 5 min each. The sections were left

Received 2/8/01; accepted 5/2/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by a grant from La Ligue to D. J. and by a United States Department of Defense Grant BCRP DAMD 17-00-1-0618 (to R. L. M.).

[2] A. M. and L. L. contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Laboratoire du Cytosquelette, Inserm U366, DBMS/CS, CEA/Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France. E-mail: djob@cea.fr.

[4] The abbreviations used are: TTL, tubulin tyrosine ligase; Glu-tubulin, detyrosinated tubulin; Δ2-tubulin, α tubulin lacking both tyrosine and glutamic acid from its COOH terminus; SBR, Scarff-Bloom-Richardson; NS, not significant.

Table 1 *Patient characteristics*

No.	134
Age (yr)	
Median	55.5
Range	25.8–85.8
Tumor-Node-Metastasis (TNM)	
Tumor size	
<20 mm	30 (22.4%)
20–40 mm	37 (27.6%)
≥40 mm	67 (50.0%)
Node involvement	
N_0	47 (35.1%)
N_1	87 (64.9%)
Metastasis	
M_0	120 (89.5%)
M_1	14 (10.4%)
Receptor status	
ER[a]+PgR+	77 (57.5%)
ER+PgR−	12 (9.0%)
ER−PgR+	6 (4.5%)
ER−PgR−	24 (17.9%)
Noninterpretable	15 (11.2%)
SBR Grade	
1	16 (11.9%)
2	66 (49.3%)
3	52 (38.8%)
Glu-tubulin grade	
1, negative	63 (47%)
2, positive	45 (33.6%)
3, strongly positive	26 (19.4%)
Δ2-tubulin grade	
1, negative	103 (77.0%)
2, positive	27 (20.0%)
3, strongly positive	4 (3.0%)
Clinical evolution[b]	
No complication	82 (61.2%)
Metastasis	32 (23.9%)
Death related to cancer	14 (10.4%)
Death not related to cancer	5 (3.7%)
Unknown	1 (0.7%)

[a] ER, estrogen receptor; PgR, progesterone receptor.
[b] For patients with complications, the most severe complication is taken into account. Node involvement and metastatic expansion were rated according to the TNM classification of the International Union against Cancer.

to cool down in the buffer at room temperature for about 20 min and then rinsed in PBS. All of the tissue sections were treated with 3% H_2O_2 for 15 min to exhaust endogenous peroxidase. The sections were then immersed in PBS containing 0.3% BSA-0.5% Triton for 6 min. After preincubation in 3% BSA in PBS for 30 min, sections were incubated overnight at 4°C with polyclonal Glu-tubulin (L3) or Δ2-tubulin (L7) antibodies. These antibodies were produced in our laboratory (13) and, beforehand, were affinity-purified against the corresponding heptapeptide. Affinity-purified L3 and L7 antibodies were used at dilutions of 1:8000 and 1:250, respectively. Sections were then rinsed 3 times in PBS-0.1% Tween and incubated, at room temperature, sequentially in biotinylated goat antirabbit secondary antibody at 1:300 dilution (30 min), and in avidin-biotin peroxidase complex (30 min; DAKO SA). Chromogenic development was obtained by adding a diaminobenzidine solution (kit DAKO SA) for 6 min. Finally, sections were slightly counterstained with Harris' hematoxylin, dehydrated, mounted in Eukitt medium, and examined by light microscopy.

Statistics. The association of variables was evaluated using the χ^2 test, the Fisher exact test, variance analysis, or the log-rank test, depending of the nature of the variables and of the group size. The value of significance was taken as $P < 0.05$. Statistical analysis was carried out using Statistica software.

RESULTS

Analysis of Glu-tubulin in Cancer Cells. Tissue sections stained with Glu-tubulin antibody were examined for presence of cells containing Glu-tubulin as described above. In normal areas of breast tissue sections, Glu-tubulin staining was detected in the cytoplasm of fibroblasts (Fig. 1*A*) and of endothelial cells. The nuclei of myoepithelial cells were occasionally stained. This nuclear labeling may result from a cross-reaction with a nuclear protein in this cell type because tubulin is normally undetectable in cell nuclei. Most importantly, normal epithelial cells were never stained (Fig. 1*A*). In contrast, Glu-tubulin was detected in transformed epithelial cells (Fig. 1, *C* and *D*). Stromal cells within the tumor were also stained. For the scoring of tumors, slides were microscopically evaluated by three observers (A. M., L. L., I. T.) without knowledge of either the prior histological results or the clinical outcome. The percentage of positively stained transformed epithelial cells in the infiltrating component were estimated by each investigator. Tumors were scored according to the percentage of positive cancer cells: grade 1, negative for Glu-tubulin staining (Fig. 1*B*); grade 2, positive, with <50% of cancer cells positive for Glu tubulin (Fig. 1*C*); and grade 3, strongly positive with ≥50% of cancer cells positive for Glu-tubulin (Fig. 1*D*). When the scores determined by the investigators differed significantly, a consensus scoring was decided by joint examination. Nerve fibers known to exhibit high levels of Glu-and Δ2-tubulin (13) were used as internal positive controls. Negative controls were obtained by omission of the primary antibody. To test the robustness of the Glu-tubulin scoring, a sample of 34 slides was scored again by an independent observer (M. H. P.), and the scoring was compared with the consensus scoring. Scores were identical in 28 cases, showing a good reproducibility of the scoring (Kendall concordance coefficient, 0.79). With this scoring, >50% of the tumors were positive for Glu-tubulin staining (Table 1). Thus, tubulin detyrosination is a common occurrence in breast cancer cells.

Other sections from the same tumor blocks as those used for Glu-tubulin scoring were stained with Δ2-tubulin antibody. These tumor sections were analyzed without knowledge of the Glu-tubulin score. Staining of normal cell types was almost identical to the staining obtained with Glu-tubulin antibody, except that the nuclear staining of myoepithelial cells was almost constant, whereas fibroblasts were only exceptionally stained (Fig. 1*E*). Normal epithelial cells were never stained (Fig. 1*E*). In contrast, Δ2-tubulin could be detected in cancer cells (Fig. 1*F*). The scoring was done as described for Glu-tubulin. Over 20% of the tumors scored positive for Δ2-tubulin staining (Table 1). Ninety % (28 of 31) of the Δ2-tubulin-positive tumors were also Glu-tubulin positive. There was a strong overall correlation between positivity for Δ2-tubulin and positivity for Glu-tubulin ($P < 0.00001$).

Relationship between Tubulin Detyrosination and Markers of Tumor Severity. Tubulin detyrosination could occur at random among breast tumors or could be related to tumor severity, as assessed by clinical and cytological markers. To test which of these possibilities was correct we analyzed the relationship between the tumor detyrosination score and known markers of tumor severity. Major prognostic factors in breast tumors include patient age, tumor size and axillary lymph node involvement (17). Cytological markers include the steroid receptor status and the so-called SBR grade. In our population, detyrosination was unrelated to age, tumor size, axillary node involvement and receptor status (Table 2). In contrast, there was a highly significant association between positivity for Glu-tubulin and high SBR grade (Table 2). The SBR grade has three components, scoring tubular differentiation, nuclear pleiomorphism, and the proportion of mitotic cells, respectively (16). Nuclear pleiomorphism was not significantly related to detyrosination (Table 2). Interestingly, detyrosination was strongly and specifically correlated with the mitotic score. In strongly detyrosinated tumors, the mitotic score was high (Table 2). The score of tubular differentiation also differed among detyrosination classes, but this was probably the result of statistical fluctuations: the score was lower in the grade 2 Glu-tubulin-positive tumors than in the two other groups (Table 2) and did not differ significantly between Glu-tubulin-negative tumors (grade 1) and strongly positive (grade 3) tumors ($P = 0.32$, NS). In contrast, the

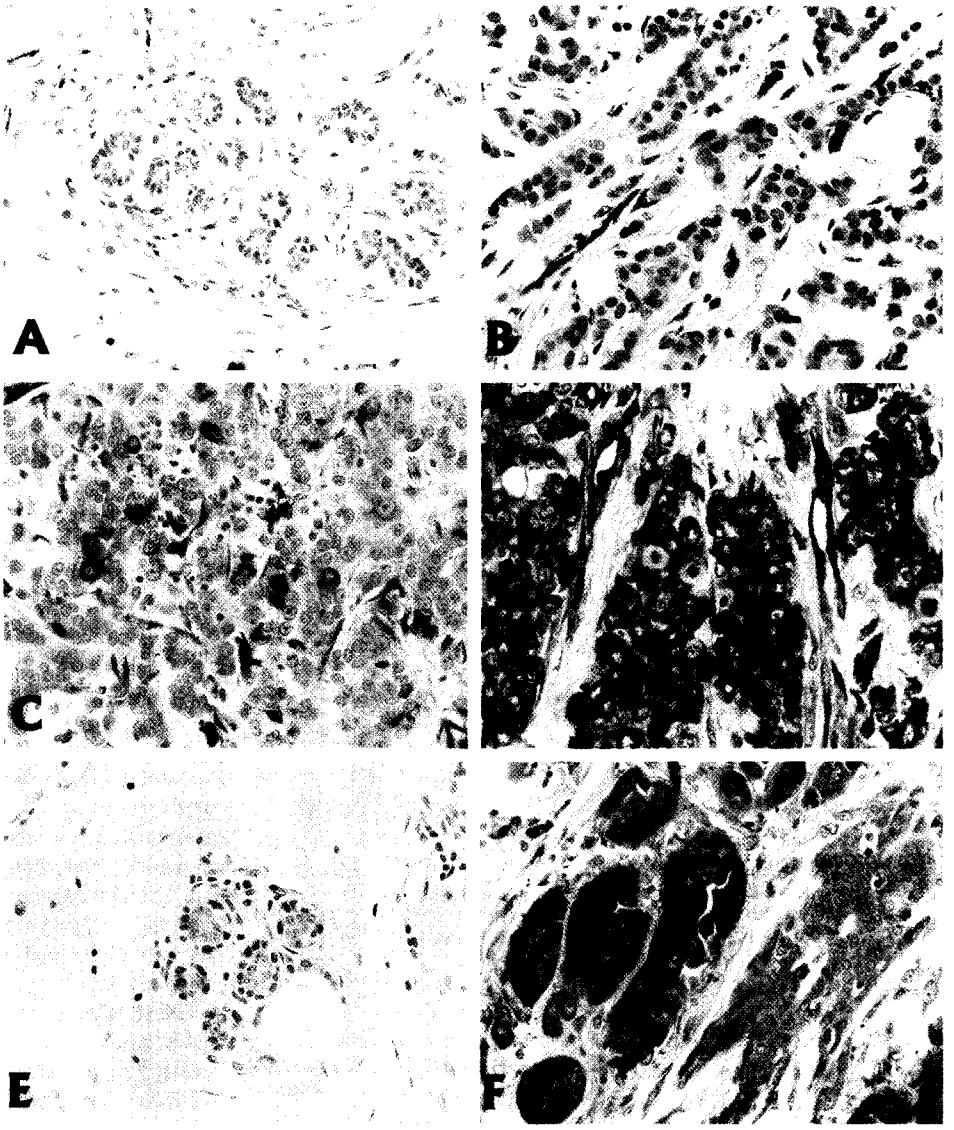

Fig. 1. Immunohistochemical staining of Glu- and Δ2-tubulin in breast tumor tissue. Paraffin-embedded breast tissue sections were stained as described in "Materials and Methods" for Glu-tubulin (*A–D*) or Δ2-tubulin (*E* and *F*). Sections were counterstained with Harris's hematoxylin. Examples of immunostaining in normal areas of breast tissue are shown for Glu-tubulin (*A*) and for Δ2-tubulin (*E*), respectively. Nuclei are *stained in blue* by hematoxylin, and Glu- or Δ2-tubulin is *stained in brown*. Examples of tumors scored as negative (grade 1), positive (grade 2), or strongly positive (grade 3) for Glu-tubulin staining are shown in *B*, *C*, and *D*, respectively. A tumor strongly positive for Δ2-tubulin is shown in *F*.

SBR grade and the mitotic score both differed significantly between the two extreme classes of negative and strongly positive ($P < 0.001$ and $P < 0.01$, respectively).

Tubulin Detyrosination and Clinical Outcome. A total of 46 patients had complications related to breast cancer (recurrence and metastasis, metastasis, death) within the period of observation. The patient selection process (see "Materials and Methods") equalized major prognostic factors such as age and tumor size among patients with or without complications. Therefore, in the studied population, neither age nor tumor size was related to the clinical outcome ($P = 0.67$ and $P = 0.32$, respectively). The relationship between the clinical outcome and both the detyrosination and SBR classes is shown in Table 3. Neither the detyrosination index nor the SBR index had significant prognostic value in our study ($P = 0.27$ and $P = 0.11$, respectively), although in both cases, a trend toward accumulation of patients with complications in the highest-grade class was observed. Could the detyrosination score be used to improve the prognostic value of the SBR grade? It is the clinician's experience that the SBR classification is nonambiguous for the extreme classes (1 and 3) but that the class 2 tumors (which are the most numerous) pose a problem, some of them being almost class 1, and others almost class 3. We tried to use the detyrosination score to reclassify the SBR2 tumors: SBR2 tumors were considered as SBR3 if they were strongly positive for Glu-tubulin (grade 3). This defined a corrected SBR index, designated SBRc. The SBRc correlated significantly with the occurrence of complications ($P = 0.02$, Table 3). The apparently improved prognostic value of SBRc compared with SBR was explained by subgroup analysis that showed that among SBR grade 2 patients, cancer complications occurred in 5 of 8 patients with a detyrosination index of 3 and in only 15 of 58 patients with lower detyrosination grades ($P < 0.05$, Fisher's exact test).

DISCUSSION

The present study shows that tubulin detyrosination is a specific and common occurrence in human breast primary tumor cells. Glu-tubulin was absent in the epithelial cells of normal breast tissue but was observed in over 50% of carcinomatous tumor cells. Δ2-tubulin accumulation was also frequent, although of somewhat more restricted occurrence than Glu-tubulin accumulation. In cultured cycling cells, Glu-tubulin levels can vary to some extent as a function of the cell cycle (10, 18, 19), whereas Δ2-tubulin has only been observed after TTL suppression (15). Therefore, in principle, Δ2-tubulin accumulation is a more specific marker of TTL suppression than Glu-tubulin accumulation. However, in the present study, Glu- and Δ2-tubulin signals were very strongly correlated, which indicated that both Δ2-tubulin accumulation and Glu-tubulin accumulation resulted

from TTL suppression. In cultured cells with suppressed TTL activity, Glu-tubulin is much more abundant than Δ2-tubulin (8, 15). Therefore, Glu-tubulin is an easier marker of TTL loss to detect than is Δ2-tubulin. This probably accounts for the excess number of Glu-tubulin-positive tumors compared with the number of Δ2-tubulin-positive tumors observed in this study. We have found Glu-tubulin scoring of tumors to be easy and reproducible among observers. This scoring should, therefore, be easy to perform in routine clinical practice and offers a unique possibility to detect the loss of a putative tumor suppressor gene (*TTL*) by the accumulation of an abnormal variant of a major cell protein (Glu-tubulin).

Our study shows that tubulin detyrosination does not occur at random among breast tumors. Instead, tubulin detyrosination is more frequent in tumors with a high SBR grade, a known marker of tumor severity (16). The detyrosination grade was apparently unrelated to the differentiation status of the tumor, as assessed by the tumor histomorphology and steroid receptor status, whereas it strongly correlated with the mitotic score. These results agree with previous studies, which indicated that the tyrosination cycle is not a differentiation marker (2, 3, 20) and that its inhibition in cancer cells somehow favors tumor growth (15).

Is tubulin detyrosination a clinically useful marker of tumor prognosis? Our preliminary data indicate that tubulin detyrosination may define high- and low-risk groups in breast cancer patients, which represent the majority of breast tumor patients, with an SBR grade of 2. A simple combination of the SBR and of the detyrosination grades apparently yielded an index with improved prognostic value. These results are encouraging but are based on the analysis of groups of small size and obviously need to be confirmed by studies of much larger patient populations. We believe that the ease of the Glu-tubulin scoring and its potential usefulness justify its evaluation in such studies. The ubiquity of the tubulin tyrosination cycle (2, 3, 20) suggests that TTL elimination may occur in several types of cancers. In a preliminary limited survey of colon and lung cancers, we have, indeed, observed Glu-tubulin and Δ2-tubulin positive tumors. Therefore, assaying tubulin detyrosination may be of clinical interest in epithelial cancers in general.

Table 2 *Relationship between tubulin detyrosination and clinical/biological characteristics of primary breast cancer*

	Tubulin detyrosination grade			
	1 (negative) (n = 63)	2 (positive) (n = 45)	3 (strongly positive) (n = 26)	P^a
Age				
< 45 yr	13 (20.6%)	11 (24.4%)	4 (15.4%)	
45–65 yr	31 (49.2%)	21 (46.7%)	10 (38.5%)	NS
≥ 65 yr	19 (30.2%)	13 (28.9%)	12 (46.1%)	
Tumor size				
< 20 mm	17 (27.0%)	10 (22.2%)	3 (11.5%)	
20–40 mm	18 (28.6%)	10 (22.2%)	9 (34.6%)	NS
≥ 40 mm	28 (44.4%)	25 (55.6%)	14 (53.8%)	
Axillary node involvement				
N_0	21 (33.3%)	16 (35.6%)	10 (38.5%)	
N_1	42 (66.7%)	29 (64.4%)	16 (61.5%)	NS
Receptor status				
ER[b]+PgR+	39 (61.9%)	25 (55.6%)	13 (50.0%)	
ER+PgR−	7 (11.1%)	4 (8.8%)	1 (3.8%)	
ER−PgR+	3 (4.8%)	3 (6.7%)	0 (0.0%)	NS
ER−PgR−	10 (15.9%)	5 (11.1%)	9 (34.6%)	
Non determined	4 (6.3%)	8 (17.8%)	3 (11.5%)	
SBR grade				
1	11 (17.5%)	4 (8.9%)	1 (3.8%)	
2	37 (58.7%)	21 (46.7%)	8 (30.8%)	<0.001
3	15 (23.8%)	20 (44.4%)	17 (65.4%)	
Differentiation score				
1	3 (4.8%)	3 (6.7%)	0 (0.0%)	
2	15 (23.8%)	17 (37.8%)	4 (15.4%)	<0.05[c]
3	45 (71.4%)	25 (55.6%)	22 (84.6%)	
Nuclear pleiomorphism score				
1	3 (4.8%)	3 (6.7%)	1 (3.8%)	
2	39 (61.9%)	17 (37.8%)	11 (4.2%)	NS
3	21 (33.3%)	25 (55.6%)	14 (53.8%)	
Mitotic score				
1	35 (55.6%)	18 (40.0%)	8 (30.8%)	
2	14 (22.2%)	10 (22.2%)	5 (19.2%)	<0.05
3	14 (22.2%)	17 (37.8%)	13 (50.0%)	

[a] *P*s correspond to one-way ANOVA considering SBR grade and scores as quantitative variables.
[b] ER, estrogen receptor; PgR, progesterone receptor.
[c] For interpretation of this result, see Text.

Table 3 *Relationship between tubulin detyrosination, SBR, SBRc, and cancer-related complications*

P correspond to Pearson χ^2. Log-rank tests gave similar results.

	Occurrence of cancer-related complications	
Tubulin detyrosination grade		
1	22/63 (34.9%)	
2	12/45 (26.7%)	P = 0.27
3	12/26 (46.2%)	
SBR grade		
1	3/16 (18.8%)	
2	20/66 (30.3%)	P = 0.11
3	23/52 (44.2%)	
SBRc		
1	3/16 (18.8%)	
2	15/58 (25.9%)	P = 0.02
3	28/60 (46.7%)	

REFERENCES

1. Jordan, M. A., and Wilson, L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr. Opin. Cell Biol., *10:* 123–130, 1998.
2. Barra, H. S., Arce, C. A., and Argarañia, C. E. Posttranslational tyrosination/detyrosination of tubulin. Mol. Neurobiol., *2:* 133–153, 1988.
3. MacRae, T. H. Tubulin post-translational modification-enzymes and their mechanisms of action. Eur. J. Biochem., *244:* 265–278, 1997.
4. Ersfeld, K., Wehland, J., Plessman, U., Dodemont, H., Gerke, V., and Weber, K. Characterization of the tubulin-tyrosine ligase. J. Cell Biol., *120:* 725–732, 1993.
5. Hallak, M. E., Rodriguez, J. A., Barra, H. S., and Caputto, R. Release of tyrosine from tyrosinated tubulin. Some common factors that affect this process and the assembly of tubulin. FEBS Lett., *73:* 147–150, 1977.
6. Webster, D. R. Tubulinyl-Tyr carboxypeptidase. *In:* A. J. Barrett, N. D. Rawlings, and J. F. Woessner (eds), Handbook of Proteolytic Enzymes, pp. 1565–1567. London: Academic Press, 1998.
7. Paturle-Lafanechère, L., Eddé, B., Denoulet, P., Van Dorsselaer, A., Mazarguil, H., Le Caer, J. P., Wehland, J., and Job, D. Characterization of a major brain tubulin variant which cannot be tyrosinated. Biochemistry, *30:* 10523–10528, 1991.
8. Lafanechère, L., and Job, D. The third tubulin pool. Neurochem. Res., *25:* 11–18, 2000.
9. Wehland, J., Willingham, M. C., and Sandoval, I. V. A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. I. Biochemical characterization, effects on microtubule polymerization and organization *in vivo*. J. Cell Biol., *97:* 1467–1475, 1983.
10. Gundersen, G. G., Kalnoski, M. H., and Bulinski, J. C. Distinct populations of microtubules: tyrosinated and nontyrosinated α tubulin are distributed differently *in vivo*. Cell, *38:* 779–789, 1984.
11. Wehland, J., and Weber, K. Turnover of the carboxy-terminal of α-tubulin and means of reaching elevated levels of detyrosination in living cells. J. Cell Sci., *88:* 185–203, 1987.
12. Kreis, T. E. Microtubules containing detyrosinated tubulin are less dynamic. EMBO J., *6:* 2597–2606, 1987.
13. Paturle-Lafanechère, L., Manier, M., Trigault, N., Pirollet, F., Mazarguil, H., and Job, D. Accumulation of Δ2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. J. Cell Sci., *107:* 1529–1543, 1994.
14. Guillaud, L., Bosc, C., Fourest-Lieuvin, A., Denarier, E., Pirollet, F., Lafanechère, L., and Job, D. STOP proteins are responsible for the high degree of microtubule stabilization observed in neuronal cells. J. Cell Biol., *142:* 167–179, 1998.
15. Lafanechère, L., Courtay-Cahen, C., Kawakami, T., Jacrot, M., Rüdiger, M., Wehland, J., Job, D., and R. L. Margolis. Suppression of tubulin tyrosine ligase during tumor growth. J. Cell Sci., *111:* 171–181, 1998.
16. Elston, C. W., and Ellis, I. O. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, *19:* 403–410, 1991.
17. Fisher, E. R., Costantino, J., Fisher, B., and Redmond, C. Pathologic findings from the National Surgical Adjuvant Breast Project (protocol 4). Discriminants for 15 years survival. Cancer (Phila.), *71:* 2141–2150, 1993.
18. Geuens, G., Gundersen, G. G., Nuydens, R., Cornelissen, F., Bulinski, J. C., and DeBrabander, M. Ultrastructural colocalization of tyrosinated and detyrosinated α-tubulin in interphase and mitotic cells. J. Cell Biol., *103:* 1883–1893, 1986.
19. Bré, M. H., Pepperkok, R., Kreis, T. E., and Karsenti, E. Cellular interactions and tubulin detyrosination in fibroblastic and epithelial cells. Biol. Cell., *71:* 149–160, 1991.
20. Preston, S. F., Deanin, G. G., Hanson, R. K., Gordon, M. W. The phylogenetic distribution of tubulin:tyrosine ligase. J. Mol. Evol., *13:* 23–44, 1979.